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Intrinsic rifamycin resistance of *Mycobacterium abscessus* is mediated by ADP-ribosyltransferase MAB_0591

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Objectives: Rifampicin, a potent first-line TB drug of the rifamycin group, shows only little activity against the emerging pathogen *Mycobacterium abscessus*. Reportedly, bacterial resistance to rifampicin is associated with polymorphisms in the target gene *rpoB* or the presence of enzymes that modify and thereby inactivate rifampicin. The aim of this study was to investigate the role of the *MAB_0591* (arr_{Mab})-encoded rifampicin ADP-ribosyltransferase (Arr_*Mab*) in innate high-level rifampicin resistance in *M. abscessus*.

Methods: Recombinant *Escherichia coli* and *Mycobacterium tuberculosis* strains expressing *MAB_0591* were generated, as was an *M. abscessus* deletion mutant deficient for *MAB_0591*. MIC assays were used to study susceptibility to rifampicin and C25 carbamate-modified rifamycin derivatives.

Results: Heterologous expression of *MAB_0591* conferred rifampicin resistance to *E. coli* and *M. tuberculosis*. Rifamycin MIC values were consistently lower for the *M. abscessus* Δarr_{Mab} mutant as compared with the *M. abscessus* ATCC 19977 parental type strain. The rifamycin WT phenotype was restored after complementation of the *M. abscessus* Δarr_{Mab} mutant with arr_{Mab}. Further MIC data demonstrated that a C25 modification increases rifamycin activity in WT *M. abscessus*. However, MIC studies in the *M. abscessus* Δarr_{Mab} mutant suggest that C25 modified rifamycins are still subject to modification by Arr_*Mab*.

Conclusions: Our findings identify Arr_*Mab* as the major innate rifamycin resistance determinant of *M. abscessus*. Our data also indicate that Arr_*Mab*-mediated rifamycin resistance in *M. abscessus* can only in part be overcome by C25 carbamate modification.

Introduction

Mycobacterium abscessus, an environmental saprophyte, is one of the most pathogenic and drug-resistant organisms among rapidly growing mycobacteria (RGM).[1,2] It accounts for ∼80% of all lung infections due to RGM, particularly exacerbations of chronic lung disease in patients with cystic fibrosis or bronchiectasis. *M. abscessus* is also responsible for skin or soft tissue infections, usually following trauma, plastic surgery or aesthetic procedures (tattooing and body piercing).[3–11] Currently, there is no reliable antibiotic regimen for the treatment of infections with *M. abscessus*, as this bacterium demonstrates a high level of intrinsic and acquired resistance to commonly administered antibiotics.[2,3,7,11]

Rifampicin is a major chemotherapeutic agent of the rifamycin group.[12,13] The rifampicin mechanism of action is based on its ability to inhibit transcription by binding with high affinity to the *rpoB*-encoded β-subunit (RpoB) of the DNA-dependent RNA polymerase of prokaryotes.[14,15] Bacterial rifampicin resistance is mostly attributed to alterations in *rpoB*, responsible for decreased affinity of the RpoB for rifampicin.[15–18] Less frequent mechanisms of rifampicin resistance include limited membrane permeability to rifampicin and enzymatic inactivation of rifampicin through modification processes.[19–24]

Rifampicin resistance mutations were originally identified and extensively studied in *Escherichia coli* and are mapped in four distinct sequence clusters within the *rpoB* gene, known as the N-terminal cluster (N) and clusters I, II and III.[25–30] It has subsequently been shown that the majority of rifampicin resistance mutations occur within an 81 bp region of cluster I, denoted as the rifampicin resistance-determining region, across all bacterial species.[28,31–33] In *Mycobacterium tuberculosis*, high-level clinically acquired rifampicin resistance is almost always conferred by mutations in *rpoB*.[34] In contrast, *Mycobacterium smegmatis*, which is naturally resistant to rifampicin, has rifampicin ADP-ribosyltransferase activity conferring innate rifampicin resistance.[22,35,36] Combrink *et al.*[37]

delineated that the mechanism of Arr_*Msm*-mediated rifampicin inactivation can be overcome by a series of 3-morpholino rifamycins in which the C25 acetate group of the rifamycin core was replaced by a bulky carbamate group. The C25 carbamate prevents ribosylation of the adjacent C23 alcohol catalysed by Arr_*Msm* and thus C25 rifamycins exhibit improved antimicrobial activity compared with rifampicin against *M. smegmatis*.[37]

WGS revealed that *M. abscessus* carries a putative ADP-ribosyltransferase (Arr_*Mab*), encoded by *MAB_0591* (arr_{Mab}).[11,38] To date, no experimental data have defined the exact function of *MAB_0591* nor confirmed its association with *M. abscessus* innate high-level rifampicin resistance.

We here used heterologous expression of *MAB_0591* and targeted deletion of *MAB_0591* in *M. abscessus* to identify Arr_*Mab* as the major relevant rifampicin resistance determinant in the type strain *M. abscessus* ATCC 19977. In addition, our finding that Arr_*Mab*-mediated rifampicin resistance in *M. abscessus* can hardly be overcome by rifamycin C25 carbamate modification has important consequences for the development of new rifamycin derivatives active against *M. abscessus*.

Materials and methods

RpoB amino acid sequence alignment

The RpoB sequences of *E. coli* K-12, *M. tuberculosis* H37Rv, *M. smegmatis* mc^2155 and *M. abscessus* ATCC 19977 were collected from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/protein/). Multiple sequence alignment was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Bacterial strains and growing conditions

(i) *E. coli* strains were cultivated in LB medium at 37 °C overnight. Ampicillin was added to the medium at a final concentration of 120 mg/L when necessary. For all cloning steps, *E. coli* XL1-Blue or *E. coli* MC1061 were used, whereas the *E. coli-gfp* control strain and *E. coli-*arr_{Mab} testing strain were used for MIC assays. (ii) *M. abscessus* strains were grown in Middlebrook 7H9 liquid medium or on LB agar plates at 37 °C for 5 days. When needed, apramycin was added at a final concentration of 50 mg/L. The *M. abscessus* ATCC 19977 type strain, *M. abscessus* Δarr_{Mab} mutant strain, *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant strain and *M. abscessus* Δarr_{Mab} pMV361-*aac(3)IV* vector backbone control strain were used throughout this study. (iii) The *M. smegmatis* mc^2155 type strain was grown on LB agar plates at 37 °C for 3 days. (iv) *M. tuberculosis* strains were cultivated in Middlebrook 7H9 liquid medium or on Middlebrook 7H10 agar plates at 37 °C for 2–4 weeks and apramycin was added at a final concentration of 50 mg/L when necessary. The *M. tuberculosis* H37Rv type strain, Mtb-*aac(3)IV* control strain and Mtb-*aac(3)IV-*arr_{Mab} testing strain were used in this study.

Antibiotics

Ampicillin, apramycin, amikacin, isoniazid, rifampicin, rifapentine and rifaximin were bought from Sigma–Aldrich, Switzerland. The following C25 modified rifamycin derivatives were synthesized according to the literature protocol:[37] (i) 5f, 25-*O*-desacetyl-(4-methoxybenzylaminocarbonyl) 3-morpholino rifamycin S; (ii) 5k, 25-*O*-desacetyl-{C-[3-(2-methoxy-phenyl)-isoxazol-5-yl]-methylaminocarbonyl} 3-morpholino rifamycin S; and (iii) 5l, 25-*O*-desacetyl-[C-(3-pyridin-2-yl-isoxazol-5-yl)-methylaminocarbonyl] 3-morpholino rifamycin S. The identity of the compounds was confirmed by NMR and MS analysis. Purity of each compound was >97% as analysed by HPLC. Compounds were dissolved in H_2O or DMSO according to the manufacturer's recommendations, were filter sterilized, aliquotted into stock solutions of 5–50 g/L and finally stored at −20 °C.

Expression of MAB_0591 in E. coli

MAB_0591 was amplified from *M. abscessus* ATCC 19977 genomic DNA by Phusion High-Fidelity DNA Polymerase PCR (5′-ATATATGCTCTTCTA GTACGATGCCCAACTTTTTGA-3′ and 5′-TATATAGCTCTTCATGCGTCA TAGATGACCGCGTTTCC-3′). Following initial cloning into the pINIT vector, the sequence-verified *MAB_0591* amplicon was inserted downstream of the arabinose-inducible pBAD promoter into the multicopy expression vector pBXNH3 via a fragment exchange cloning system.[39] The resulting *E. coli* MC1061 pBXNH3-arr_{Mab} testing strain is referred to as *E. coli-*arr_{Mab}. As a control, *gfp* was amplified from the pOLYG-*gfp-hyg* vector (5′-ATATATGCTCTTCTAGTATCTCGAAGGGCGAGGAGCT-3′ and 5′-TATATA GCTCTTCATGCCTTGTACAGCTCGTCCATGCCG-3′) and cloned directly into the pBXNH3 expression vector. The resulting *E. coli* MC1061 pBXNH3-*gfp* control strain expressing *gfp* is referred to as *E. coli-gfp*. Gene expression of *MAB_0591* and *gfp* was conducted by induction of 30 mL bacterial cultures (OD_{600} = 0.85–1) with L-arabinose at a final concentration of 0.2% v/v, at 37 °C for 4.5 h.

Expression of MAB_0591 in M. tuberculosis

MAB_0591 (including its native promoter) was PCR amplified from *M. abscessus* ATCC 19977 genomic DNA using KpnI-linker modified primers 5′-AGGGTACCCGGATATGTGCAGCGGCATG-3′ and 5′-GAGGTACCCACCGAAG CACTGAAGGTGC-3′ and cloned into the KpnI site of the pMV361-*aac(3)IV* vector to result in the pMV361-*aac(3)IV-MAB_0591* complementing vector. The control backbone vector [pMV361-*aac(3)IV*] and the complementing vector [pMV361-*aac(3)IV-MAB_0591*] were transformed into the electrocompetent *M. tuberculosis* H37Rv type strain, as previously described for *Mycobacterium bovis* BCG.[40] Briefly, 400 μL of *M. tuberculosis* H37Rv competent cells were mixed with 1 μg of supercoiled plasmid DNA and electroporated in a Bio-Rad Gene Pulser II (settings: 2.5 kV, 1000 Ohms and 25 μF). Following electroporation, cells were resuspended in 4 mL of 7H9-OADC-Tween 80 and incubated for 20 h at 37 °C. Appropriate dilutions were plated on selective agar and after 3 weeks of incubation, single colonies were picked, restreaked and grown in liquid broth when necessary. The Mtb-*aac(3)IV* control strain and the Mtb-*aac(3)IV-*arr_{Mab} testing strain were obtained by positive selection on 7H10 plates containing apramycin. The presence of the *aac(3)IV* and/or the *MAB_0591* gene(s) in these strains was confirmed by colony PCR.

Deletion of MAB_0591 in M. abscessus

An 1.3 kbp PscI/NotI fragment from position 591427 to 592720 (5′arr_{Mab} flanking sequence) and an 1.3 kbp NotI/XbaI fragment from position 593015 to 594322 (3′arr_{Mab} flanking sequence) were PCR amplified using genomic DNA from *M. abscessus* ATCC 19977 [(5′-GAAATTACATGT GTCACGATCTCCTGGACTGCCTC-3′, 5′-GAAAGCGGCCGCCATGGAAGTACGCA CCCGATTCG-3′) and (5′-GATAGCGGCCGCCCGAATTCATGGAAACCTTCCGGG-3′, 5′-GTCTAGAGTCCTGTGTGAACAGGTCGGTG-3′), respectively)] and stepwise cloned into the pSE-*katG-aac(3)IV* suicide vector resulting in the knockout vector pSE-*katG-aac(3)IV-*Δ*MAB_0591*. Details on the construction of the *M. abscessus* deletion mutant will be described elsewhere (A. Rominski, P. Selchow and P. Sander, unpublished results). Briefly, pSE-*katG-aac(3)IV-*Δ*MAB_0591* was transformed into electrocompetent *M. abscessus* ATCC 19977. For electroporation, 100 μL of competent cells were mixed with 1–2 μg of supercoiled plasmid DNA and electroporated in a Bio-Rad Gene Pulser II (settings: 2.5 kV, 1000 Ohms and 25 μF). After electroporation, cells were resuspended in 0.9 mL of 7H9 medium and incubated for 5 h with constant shaking (1000 rpm) at 37 °C. Appropriate dilutions were subsequently plated on selective agar and after 5 days of incubation, single colonies were picked, restreaked and grown in liquid broth when necessary.

Transformants were selected on LB agar plates containing apramycin and identified by *aac(3)IV* PCR. Single crossover transformants were identified by Southern blot analysis with a 0.2 kbp EcoRI 5′arr_{Mab} DNA probe (the same probe was also used for all subsequent Southern blot analyses) and subjected to counterselection on LB agar plates containing isoniazid (32 mg/L). Single colonies were screened for deletion of *MAB_0591* by PCR and the genotype was finally confirmed by Southern blot analysis. In this way, a 0.3 kbp region of the *MAB_0591* was deleted. For complementation of the *M. abscessus* Δarr_{Mab} mutant with *MAB_0591*, the previously described complementation vector pMV361-*aac(3)IV-MAB_0591* was transformed into the Δarr_{Mab} mutant strain. Transformation was verified by Southern blot analysis.

Susceptibility testing of M. tuberculosis strains

Drug susceptibility testing (DST) was performed using the MGIT 960 system as recommended by the manufacturer[41] and the results were interpreted as described previously by Springer *et al.*[42] Briefly, 0.5 mL dilutions of positive MGIT vials of the Mtb-*aac(3)IV* control strain and the Mtb-*aac(3)IV-*arr_{Mab} testing strain were inoculated into fresh vials containing constant levels of apramycin for plasmid maintenance and different concentrations of the test drugs.[43] Rifampicin was tested at concentrations of 1, 4 and 20 mg/L, while the control drug isoniazid was tested at concentrations of 0.1, 1, 3 and 10 mg/L. For the drug-free growth control, the bacterial working suspension was diluted 1:100 with sterile NaCl and 0.5 mL of the diluted working suspension was inoculated into the tube (proportion testing).[44] All MGIT tubes were incubated in the MGIT 960 instrument and monitored using EpiCenter (version 5.53) software equipped with the TB eXiST module (Becton Dickinson).

Rifampicin Etest

Bacterial suspensions of *M. abscessus* strains ATCC 19977, Δ$arr_{Mab}$and Δarr_{Mab}-arr_{Mab} were adjusted to a turbidity equivalent to that of a 0.50 McFarland standard and subsequently spread on LB agar plates using a sterile cotton swab. Then, a rifampicin Etest strip (bioMérieux, Switzerland) was placed on each plate and plates were incubated for 5 days at 37 °C. The point of intersection between bacterial growth and the Etest device was read as the MIC value.

MIC assays

MIC assays were performed according to CLSI guidelines.[45] Working solutions were prepared by diluting the antibiotic stock solutions in CAMHB (pH 7.4) (Becton Dickinson, Switzerland) to a concentration corresponding to twice the desired final concentration [working solutions of 128, 512 and 1024 mg/L were prepared when the highest concentrations tested in the MIC assay were 64, 256 and 512 mg/L, respectively (Table 1 and Table 3)]. By using CAMHB in sterile 96-well microtitre plates (Greiner Bio-One, Switzerland), 2-fold serial dilutions of the working solutions were prepared. A positive growth control lacking antibiotic and a sterile negative control containing only CAMHB were included in each 96-well microtitre plate. For the preparation of the inoculum, three to four colonies from each bacterial strain grown on LB agar were transferred into a glass tube containing 2 mL of NaCl using a sterile cotton swab. In order to achieve a final inoculum titre of $1–5 \times 10^5$ cfu/mL, all bacterial suspensions were adjusted to a turbidity equivalent to that of a 0.50 McFarland standard and subsequently diluted in CAMHB. The final test volume in each well of the microtitre plate was 0.1 mL. The correct titre of each inoculum was checked by obtaining cfu counts on LB agar plates. All microdilution plates were capped with adhesive sealing covers and incubated at 37 °C for (i) 16 h for *E. coli* strains and (ii) 3, 5, 7 and 12 days for *M. abscessus* and *M. smegmatis* strains, before the MIC values were assessed by visual inspection. All MIC assays were conducted in triplicate.

Table 1. DST results of *E. coli* expressing *MAB_0591*[a]

Strain	Rifampicin MIC (mg/L)
E.coli-gfp	4
E.coli-arr_{Mab}	>512

[a]Broth microdilution method

Results and discussion

Analysis of the RpoB rifampicin resistance sequence clusters from M. abscessus

To investigate whether innate rifampicin resistance in *M. abscessus* (MIC: 128 mg/L) is associated with polymorphisms in the RNA polymerase β-subunit, we aligned the RpoB amino acid sequence of *M. abscessus* ATCC 19977 with those of *E. coli* K-12 and *M. tuberculosis* H37Rv, known to be naturally susceptible to rifampicin,[13,16] and *M. smegmatis* mc^2155 that has no polymorphism in its *rpoB* gene corresponding to any known rifampicin resistance genotype,[35] but is naturally resistant to rifampicin through ADP-ribosylation of rifampicin.[22,36] Our analysis revealed no polymorphism known to confer rifampicin resistance within all four (N, I, II and III) rifampicin resistance sequence clusters of *M. abscessus* RpoB (Figure 1), suggesting that other mechanisms are involved in the high intrinsic rifampicin resistance of *M. abscessus*. Possible mechanisms include efflux of rifampicin, diminished uptake of rifampicin and enzymatic degradation or modification of rifampicin.[19–23] The latter resistance mechanism has been studied in *M. smegmatis* and *Legionella pneumophila*.[22,23] A recent genome analysis suggested that *M. abscessus* carries a putative rifampicin ADP-ribosyltransferase (Arr_*Mab*),[38] encoded by *MAB_0591* (arr_{Mab}). MAB_0591 has 66% amino acid sequence identity with the *M. smegmatis* ADP-ribosyltransferase that modifies rifampicin. However, Table S1 (available as Supplementary data at *JAC* Online), which lists the MIC values for selected mycobacterial standard strains[42,46,47] as well as information about the presence or absence of ADP-ribosyltransferase-like protein in these species, shows that a direct correlation between rifampicin MIC and the presence or absence of ADP-ribosyltransferase-like proteins does not exist. *M. tuberculosis* and *Mycobacterium leprae* were predicted to have no ADP-ribosyltransferase-like proteins and are susceptible to rifampicin (MIC: <1 mg/L).[42,47] ADP-ribosyltransferases were predicted to be present in *M. abscessus* (*M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*) and *Mycobacterium fortuitum* and these species are resistant (MIC: ≥64 mg/L).[46] In contrast, the presence of putative ADP-ribosyltransferases in e.g. *Mycobacteriumphlei*, *Mycobacterium gilvum* and *Mycobacterium marinum* does not correlate with high rifampicin MIC levels (MIC: <0.5 mg/L).[46] Therefore, a functional role of ADP-ribosyltransferases in rifampicin resistance has to be addressed experimentally. By using ADP-ribosyltransferase of *M. smegmatis* mc^2155 in a BLASTP search, we identified also other, non-mycobacterial species predicted to possess ADP-ribosyltransferase-like proteins (Table S2). Interestingly, mycobacterial ADP-ribosyltransferases show homology to the catalytic domain of exotoxin A from *Pseudomonas aeruginosa*,[48,49]

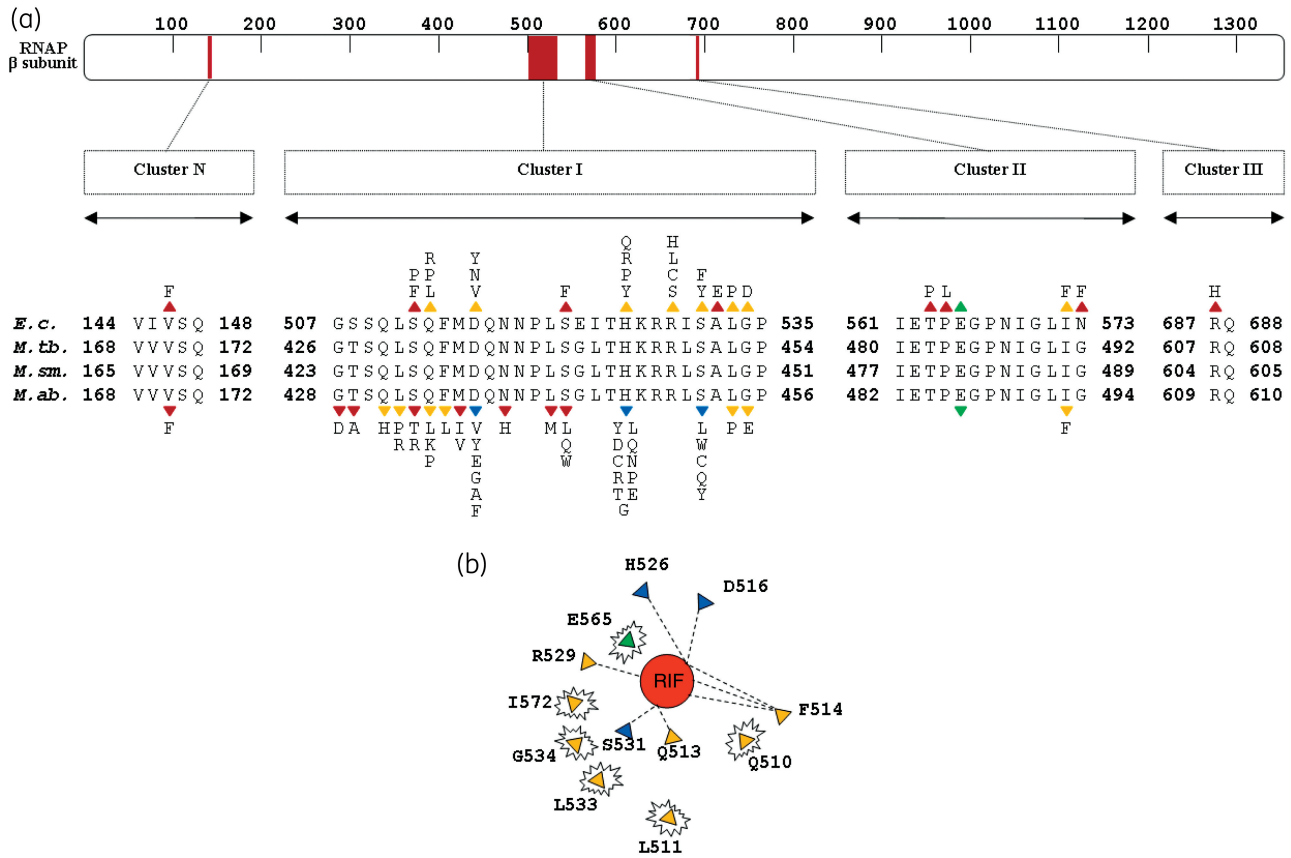

Figure 1. Rifampicin resistance regions of RpoB and their association with the interactions between rifampicin and the prokaryotic RNA polymerase (RNAP). (a) The bar at the top illustrates the RNAP β-subunit from *E. coli*. Directly above, its amino acid numbering is indicated. Dark red lines within the bar indicate the positions of the four clusters [N-terminal (N) and clusters I, II and III (I, II and III)] where the rifampicin resistance-conferring alterations are identified across all bacterial species.[24–30] Directly below follows the amino acid sequence alignment spanning the rifampicin resistance regions of *E. coli* K-12, *M. tuberculosis* H37Rv, *M. smegmatis* mc^{2}155 and *M. abscessus* ATCC 19977 RpoB. Amino acid substitutions that confer rifampicin resistance are shown as coloured triangles above and below the alignment, respectively. The possible substitutions for each position are mentioned in single amino acid code in columns directly above (for *E. coli*) or below the coloured triangles (for *M. tuberculosis*). The yellow triangles represent the residues that interact directly with rifampicin binding. In contrast, the red triangles indicate residues that are positioned too far away to have direct interaction with rifampicin. The blue triangles show the positions that are substituted with the highest frequency among *M. tuberculosis* rifampicin-resistant isolates.[31] The green triangles show a residue that is directly interacting with rifampicin, but no substitutions have been reported at this position, probably because they would be fatal for the bacterium.[15] (b) Using the same colour code for the amino acid positions as above, the residues of direct interaction with rifampicin are shown (yellow, blue and green triangles). Hydrogen bonds formed between an RpoB residue and rifampicin are depicted as broken lines and residues that form van der Waals interactions are indicated in zigzag circles. Numbering of the residues is according to *E. coli* positions.[15] RIF, rifampicin.

pointing to a putative role of mycobacterial ADP-ribosyltransferases as virulence factors. However, within this study we exclusively focus on the role of *MAB_0591* in rifamycin resistance.

Heterologous expression of MAB_0591 in E. coli and M. tuberculosis

To study if rifampicin resistance in *M. abscessus* is due to *arr$_{Mab}$*, we cloned *MAB_0591* and expressed the recombinant protein in two rifampicin-susceptible hosts, *E. coli*[16] and *M. tuberculosis*.[50] First, we addressed the question whether induced expression of *MAB_0591* would confer rifampicin resistance in a non-mycobacterial heterologous host. Arr_*Mab* was recombinantly expressed by the multicopy pBXNH3-*arr$_{Mab}$*vector in *E. coli* MC1061 under the control of the L-arabinose-inducible pBAD promoter. The *E. coli-gfp* control strain and the *E. coli-arr$_{Mab}$* testing strain were generated following transformation with the pBXNH3-*gfp* and pBXNH3-*arr$_{Mab}$* vectors, respectively. The recombinant strains were subsequently tested

Table 2. DST results of *M. tuberculosis* expressing *MAB_0591*[a]

Strain	Rifampicin 1.0 mg/L	Rifampicin 4.0 mg/L	Rifampicin 20.0 mg/L	Isoniazid 0.1 mg/L
Mtb-*aac(3)IV*	S	S	S	S
Mtb-*aac(3)IV-arr$_{Mab}$*	R	R	R	S

S, susceptible; R, resistant.
[a]Proportion method using the MGIT 960 system.

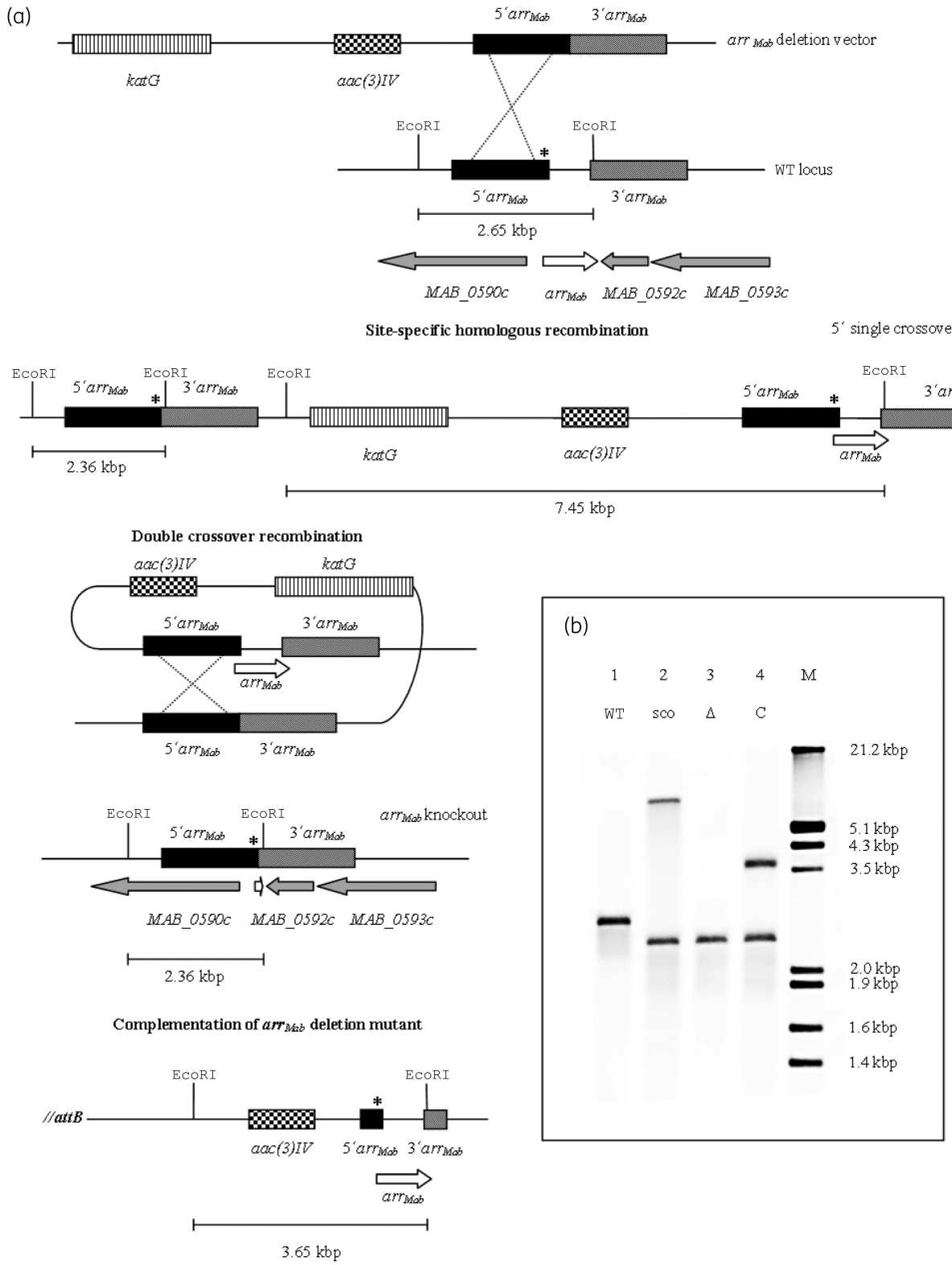

Figure 2. Genotypic analyses of the *M. abscessus* arr_{Mab}locus. (a) Schematic drawing of genotypes and recombination events. (b) Southern blot analysis confirms the deletion of *MAB_0591* from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (1), *M. abscessus* transformant with arr_{Mab} targeting vector [pSE-*katG-aac(3)IV-*Δ*MAB_0591*] prior to (2) and after KatG-dependent isoniazid counterselection (3) and after transformation of counterselected mutant with arr_{Mab} complementation vector (4) was digested with EcoRI and probed with a fragment from the 5′arr_{Mab} flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 2.65 kbp fragment of the WT parental strain, to the 2.36 and 7.45 kbp fragments after site-specific homologous recombination [single crossover (sco)], to a 2.36 kbp fragment of the Δarr_{Mab} mutant (Δ) and to the 2.36 and 3.65 kbp fragments of the *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant strain (C). M, molecular marker.

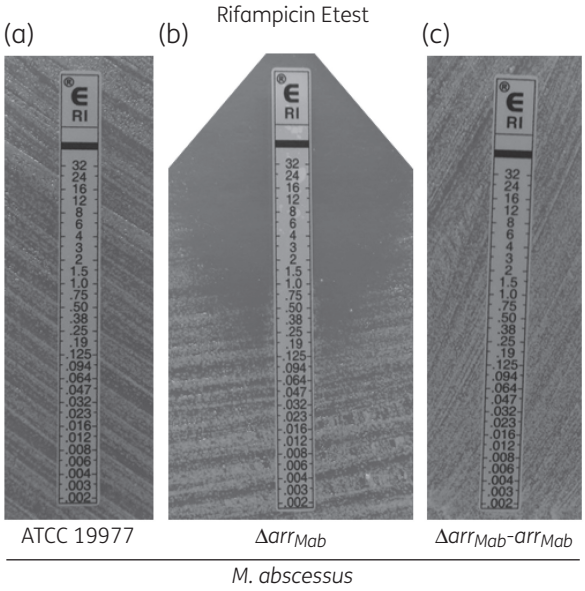

Figure 3. Contribution of *MAB_0591* to rifampicin resistance in *M. abscessus*. Rifampicin susceptibility of the (a) *M. abscessus* ATCC 19977 WT strain, (b) *M. abscessus* Δ*arr*$_{Mab}$ mutant and (c) *M. abscessus* Δ*arr*$_{Mab}$-*arr*$_{Mab}$ complemented mutant strain was examined by Etest. The picture of the rifampicin Etest results was taken after 5 days of incubation at 37 °C.

against rifampicin in MIC assays. The *E. coli-arr*$_{Mab}$ testing strain was highly resistant to rifampicin with MIC values >512 mg/L, while the *E. coli-gfp* control strain remained susceptible to rifampicin (MIC: 4 mg/L) (Table 1). These results confirm that Arr_*Mab* plays a critical role as a rifampicin resistance determinant when expressed in the rifampicin-susceptible *E. coli* host. We hypothesize that Arr_*Mab* modifies rifampicin by ADP-ribosylation at the hydroxyl group of C23, as previously shown for Arr_*Msm*,[48] and that this modification interferes with target binding.

Next, we studied whether *arr*$_{Mab}$ confers rifampicin resistance in a mycobacterial host, *M. tuberculosis*. In order to simulate veritable levels of Arr_*Mab* expression, we expressed MAB_0591 under control of its native promoter. The single-copy integrating plasmids pMV361-*aac(3)IV* and pMV361-*aac(3)IV-MAB_0591* were engineered and transformed into the rifampicin-susceptible *M. tuberculosis* H37Rv strain (MIC: <1 mg/L).[42] The *aac(3)IV* gene that confers resistance to apramycin was used as a positive selection marker. The resulting strains referred to as Mtb-*aac(3)IV* control strain and Mtb-*aac(3)IV-arr*$_{Mab}$ testing strain, respectively, were subjected to DST using the MGIT 960 system equipped with EpiCenter TB eXiST software. Susceptibility to different concentrations of rifampicin (1, 4 and 20 mg/L) and the non-substrate control drug isoniazid (0.1, 1, 3 and 10 mg/L) was determined. The Mtb-*aac(3)IV* control strain was, as expected, susceptible to both rifampicin and isoniazid at all concentrations tested (MIC: rifampicin <1.0 mg/L; isoniazid <0.1 mg/L). In contrast, the Mtb-*aac(3)IV-arr*$_{Mab}$ testing strain was specifically resistant to rifampicin, even at the highest concentration tested (MIC: >20 mg/L), but remained susceptible to the unrelated control drug isoniazid (MIC: <0.1 mg/L) (Table 2). These findings demonstrate that *MAB_0591* confers high-level resistance

Table 3. DST results of *M. abscessus* and *M. smegmatis* strains[a]

	Strain and day, MIC (mg/L)																			
	M. abscessus ATCC 19977				*M. abscessus* Δ*arr*$_{Mab}$				*M. abscessus* Δ*arr*$_{Mab}$-*arr*$_{Mab}$				*M. abscessus* Δ*arr*$_{Mab}$ pMV361-*aac(3)IV*				*M. smegmatis* mc^{2}155			
Antibiotic	3	5	7	12	3	5	7	12	3	5	7	12	3	5	7	12	3	5	7	12
Rifampicin	128	256	256	>256	0.25	0.5	1	1	128	256	256	>256	0.25	0.5	1	1	128	128	128	128
Rifapentine	256	>256	>256	>256	1	2	2	4	128	256	>256	>256	1	2	2	4	64	128	128	128
Rifaximin	64	128	256	256	1	2	4	8	64	128	256	>256	1	2	4	4	256	256	>256	>256
5f	8	>64	>64	>64	0.016	0.063	0.063	0.25	8	64	>64	>64	0.016	0.125	0.125	0.25	0.125	0.25	0.25	0.5
5k	4	32	>64	>64	0.063	0.125	0.25	0.25	4	16	>64	>64	0.031	0.125	0.25	0.25	0.25	0.25	1	1
5l	2	4	4	8	0.0078	0.125	0.25	0.25	2	4	4	4	0.016	0.125	0.25	0.25	0.25	0.5	1	1
Amikacin	1	2	4	4	1	2	4	4	1	2	4	4	1	2	4	4	<0.5	<0.5	<0.5	<0.5

[a]Broth microdilution method.

to rifampicin, but not to other drugs, when expressed in a rifampicin-susceptible mycobacterial host.

Generation of M. abscessus MAB_0591 deletion mutant

Heterologous expression of *MAB_0591* indicated that arr_{Mab} is able to confer rifampicin resistance to a susceptible host; however, its role in innate rifampicin resistance in *M. abscessus* remained to be determined. We recently developed tools for genetic manipulation of *M. abscessus* (A. Rominski, P. Selchow and P. Sander, unpublished results) and wished to exploit this technique to generate an *M. abscessus MAB_0591* deletion mutant. This mutant would allow us to directly address the role of MAB_0591 in innate rifampicin resistance. The arr_{Mab} deletion mutant was constructed by transformation of *M. abscessus* ATCC 19977 with suicide plasmid pSE-*katG-aac(3)IV-ΔMAB_0591* applying apramycin positive selection[51] and a *katG*-dependent isoniazid counterselection strategy that we previously established (A. Rominski, P. Selchow and P. Sander, unpublished results) (Figure 2a). Deletion of *MAB_0591* was confirmed by Southern blot analysis (Figure 2b). A complemented mutant strain was constructed by transformation of the *M. abscessus* Δarr_{Mab} mutant with the complementation vector pMV361-*aac(3)IV-MAB_0591* expressing Arr_*Mab*. The complemented mutant strain is referred to as *M. abscessus* Δarr_{Mab}-arr_{Mab}. Genetic complementation was confirmed by Southern blot analysis (Figure 2b).

DST of M. abscessus ΔMAB_0591

For determination of the Δarr_{Mab} mutant's phenotype, a rifampicin Etest was carried out with *M. abscessus* ATCC 19977, *M. abscessus* Δarr_{Mab} mutant and *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented strain. Etest results after 5 days of incubation at 37 °C revealed high-level rifampicin resistance in *M. abscessus* ATCC 19977 (MIC: >32 mg/L). In contrast, the Δarr_{Mab} mutant showed susceptibility to low rifampicin concentrations (MIC: ∼0.5 mg/L). Rifampicin resistance was restored upon complementation of the Δarr_{Mab} mutant strain with arr_{Mab} (MIC: >32 mg/L; Figure 3). MIC values were subsequently determined in detail for rifampicin, rifapentine and rifaximin. *M. abscessus* ATCC 19977, *M. abscessus* Δarr_{Mab}, *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant, *M. abscessus* Δarr_{Mab} pMV361-*aac(3)IV* vector backbone control strain and *M. smegmatis* mc^2155 were subjected to DST. For *M. abscessus* Δarr_{Mab}, the MICs of all rifamycins were consistently and significantly lower than for the *M. abscessus* ATCC 19977 WT strain, indicating that all tested rifamycins are modified by

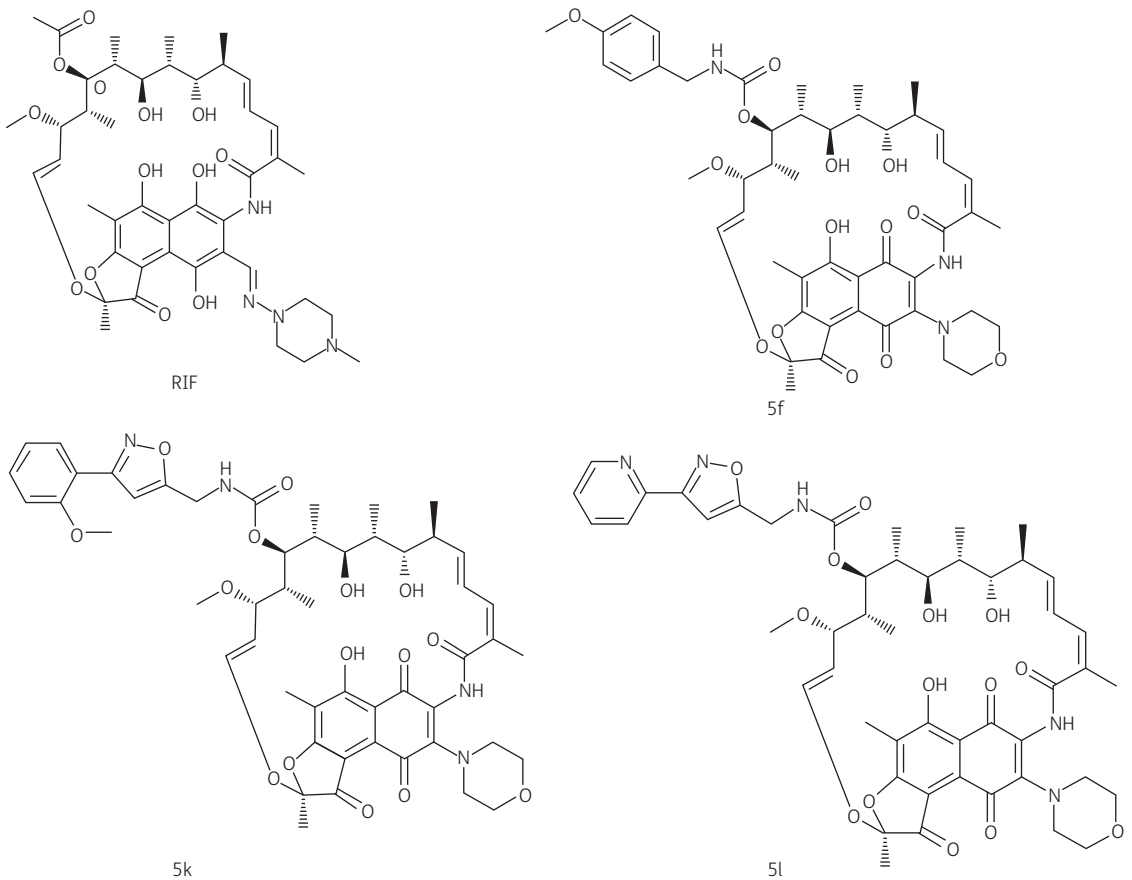

Figure 4. Rifampicin and the 5f: 25-*O*-desacetyl-(4-methoxybenzylaminocarbonyl) 3-morpholino rifamycin S, 5k: 25-*O*-desacetyl-{C-[3-(2-methoxyphenyl)-isoxazol-5-yl]-methylaminocarbonyl} 3-morpholino rifamycin S and 5l: 25-*O*-desacetyl-[C-(3-pyridin-2-yl-isoxazol-5-yl)-methylaminocarbonyl] 3-morpholino rifamycin S, C25 carbamate rifamycin derivatives, which are numbered per the original work[37] for ease of comparison. RIF, rifampicin.

Arr_*Mab* (Table 3). Transformation of the *M. abscessus* Δarr_{Mab} mutant with arr_{Mab} restored WT levels of rifamycin resistance while transformation with the empty vector backbone did not. Expectedly, amikacin MICs were independent of the *arr* genotype. These findings identify Arr_*Mab* as the major rifamycin resistance determinant in *M. abscessus*.

DST of C25 modified rifamycin derivatives

Carbamate modification at the C25 position of the rifamycin core has been shown to improve antimicrobial activity against *M. smegmatis* mc^{2}155.[37] We wanted to test whether these compounds also overcome rifamycin resistance in *M. abscessus*. C25 modified rifamycin derivatives 5f, 5k and 5l (Figure 4) were custom synthesized and tested for antimicrobial activity. The compounds showed potent activity against *M. smegmatis*—on average these compounds were 100–200-fold more active than rifampicin. These results confirm former findings that C25 modified rifamycins apparently are resilient to modification by Arr_*Msm*. The C25 modified rifamycins also showed increased activity in *M. abscessus* as compared with rifampicin, rifapentine and rifaximin (Table 3). Of note, C25 modification not only increases rifamycin activity against the *M. abscessus* WT, but also against the *M. abscessus* Δarr_{Mab} mutant, although to a lesser extent. These data indicate that the increased activity of C25 rifamycin derivatives is only partially due to resilience to Arr_*Mab* modification. Compared with compounds 5f and 5k, we observed little time-dependent increase in the MIC values of compound 5l for WT *M. abscessus* and the *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant, indicating that 5l is probably least modified by Arr_*Mab*, but still WT MIC values are high (4 mg/L; 16-fold higher than against the Δarr_{Mab} mutant).

Conclusions

Taken together, our study identified Arr_*Mab* as the major determinant of innate rifamycin resistance in *M. abscessus*. Our data indicate significant species-specific differences in rifamycin C25-mediated resilience for Arr_*Msm* and Arr_*Mab*, since Arr_*Mab*-mediated rifamycin resistance can only partly be overcome by C25 modification. These findings testify to the need to develop novel compounds that are able to escape Arr_*Mab*-mediated rifamycin resistance in *M. abscessus*. Structural similarity between Arr_*Msm* and protein ADP-ribosyltransferases, prominently domain III of *P. aeruginosa* exotoxin A, have been described.[48,49] These structural features are also conserved in Arr_*Mab*. Therefore, it is tempting to speculate on a dual role of Arr_*Mab* in drug resistance and virulence.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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